DEVELOPMENT OF COMBINATORIAL BACTERIA FOR METAL AND RADIONUCLIDE BIOREMEDIATION

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The grant concerned chromate [Cr(VI)] bioremediation and it was our aim from the outset to construct individual bacterial strains capable of improved bioremediation of multiple pollutants and to identify the enzymes suited to this end. Bacteria with superior capacity to remediate multiple pollutants can be an asset for the cleanup of DOE sites as they contain mixed waste. I describe below the progress made during the period of the current grant, providing appropriate context.

1. Chromate toxicity. Reduction of chromate is toxic to bacteria, as is illustrated in Fig. 1 [1, 2] for *P. putida* and *E. coli*: their growth is markedly inhibited and the cells acquire abnormal shapes. The external accumulation shown in *P. putida* cell (top right) have also been reported in *Caulobacter crescentus* grown in the presence of uranyl [3].

2. Soluble "chromate reductases". Some bacteria might be able to respire chromate for energy generation. This has been suggested for *Enterobacter cloacae* and might be true of others [4]. The electron transport chains of iron and sulfate reducing bacteria can also reduce chromate and other metals and radionuclides but no energy is evidently derived from many of these processes [5, 6].

We found that all of some twelve different bacteria we tested reduced chromate using *soluble* enzymes [1]. These included species of *Pseudomonas*, *Shewanella*, *Deinococcus* and *Bacillus*. *P. fluorescens*, *S. oneidensis*, *D. radiodurans* and *Vibrio harveyi* were among the most active (114 – 193 nmol chromate converted/mg cell protein/h). *Soluble* enzymes for chromate reduction have the potential advantage in that the locus for reduction is intracellular which may promote immobilization and minimize re-oxidation of the reduced species.

3. Soluble one-electron chromate reducers. Why is Cr(IV) toxic to bacteria? Several metabolic enzymes of the cell, such as lipoyl dehydrogenase, glutathione and cytochrome b_5 reductases, with the physiological role of energy generation and biosynthesis, can vicariously reduce chromate. Using electron spin resonance (ESR**),** Shi & Dalal [7] showed that such enzymes reduce chromate by one electron reduction, generating Cr(V). This is a highly reactive radical, and rapidly transfers its electron to molecular oxygen or, depending on the conditions to another molecule, generating reactive oxygen species (ROS) or other equally destructive oxidizing agents. Continual activity of the one electron reducers causes Cr(VI) to shuttle back and forth between the VI and V valence states, generating little net reduced Cr but large amounts of ROS; this drains the cell's reducing power, and damages its macromolecules through oxidation.

We tested this hypothesis *in vitro* using pure lipoyl dehydrogenase by our 'redox balance' method. This quantifies the portion of electrons donated by the reductant (NADH) to chromate and to dioxygen). While only 24% of the electrons were consumed in reducing chromate, over 70% were used in ROS generation [8, 9]. That chromate reduction by this enzyme involves extensive redox cycling is thus confirmed..

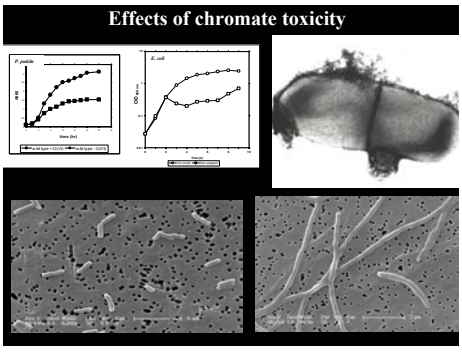

Figure 1. Growth inhibition and morphological changes (right images) generated by chromate.

4. In vivo effects of chromate reduction. Thus, certain bacterial enzymes can generate large amounts of ROS from chromate, producing little net conversion of Cr(VI) to CR(III). But does this in fact happen inside the cell? To test this we used the dye 2', 7'-dihydrodichlorofluorescein (H_2DCFDA), which is taken up by the cells and emits green fluorescence in the presence of ROS. The results (Fig. 2) [2] confirmed that cells experienced oxidative stress during chromate reduction. Further

indications of *in vivo* oxidative stress by chromate are: a) Cellular free thiols and glutathione levels decline; proteins involved in replenishing the thiol pools (CysN, CysK, sulfate adenylyl transferase) are upregulated, as is SodB that decomposes O^{-} [10-12]. b) Mutants strains missing the individual antioxidant defense genes, *cysK*, *sodB*, *katE* and *yieF*, showed greater sensitivity to chromate than the wild type. And c) the *sfiA* gene which is signature gene for the induction of the SOS response due to oxidative-stress is induced. Temporal analysis showed that the up-regulation of these proteins coincided with partial recovery of the cells from chromate stress (decreased H_2DCFDA fluorescence; return to more normal cell morphology) [2].

Oxidative stress is of course only a part of chromate stress. Cr(III), the desirable product of chromate reduction for bioremediation, is itself toxic [13]. However, from the perspective of using bacteria in remediating chromate, the difference between ROS and Cr(III) toxicity is critical. The former damages bacteria before they have had the chance to carry out the desired reaction; the latter does so after, and there may be a significant chance that a dead bacterium with Cr(III) complexed within it affords a means of immobilization. Thus, it would appear that minimizing chromate-mediated oxidative stress is critical for improving bacterial bioremediation capacity.

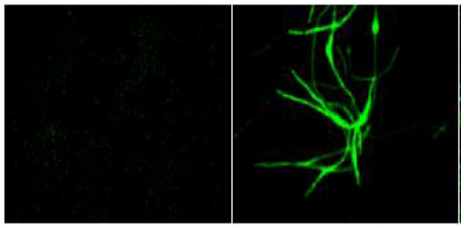

Figure 2. Chromate engendered oxidative stress makes the dye fluoresce (right panel)

5. Obligate two-electron reducers. How can oxidative stress engendered by chromate be minimized? Guidance on this came from studies in mammalian cells. They contain an enzyme, called DT diaphorase (NQO1), which is an obligatory two-electron reducer of its substrates. We hypothesized that if bacteria possessed a functional homologue of this enzyme then that enzyme could, as dimer, reduce chromate in one step. This would bypass Cr(V) generation, avoid redox cycling and generate Cr(III) with minimal ROS generation. Such a bacterial enzyme if made more active would outcompete the cellular one-electron chromate reducers and minimize chromate toxicity to the remediating bacteria.

Using a variety of approaches, we succeeded in purifying to homogeneity one such enzyme (ChrR) from *P. putida* [14]. Analyses showed that several bacteria possessed its sequence orthologues (~30%, amino acid identity), but no function assignment had been made to any of these proteins [15]. We have cloned the genes and extensively characterized two bacterial enzymes of this class, ChrR and YieF, as well as an unrelated enzyme (sequence-wise), NfsA, which has been extensively studied [16] but its capacity for obligatory two-electron reduction of chromate was not known prior to our work [9, 15]. Of these YieF was found to be the most tight obligatory two-electron chromate reducer with the widest substrate range, and was therefore chosen for further study.

That YieF reduces chromate without involving redox cycling was confirmed using multiple experimental approaches. The redox balance method showed that only 25% of the electrons donated by NADH were consumed in ROS generation, the rest being utilized in chromate reduction. Thus, the four-electron reduced YieF dimer catalyzed a one-step three electron reduction of Cr(VI) to Cr(III), with the remaining electron simultaneously reacting with dioxygen to generate ROS, bypassing Cr(V) generation and redox cycling. That Cr(V) generation in fact did not occur was confirmed by rapid scan spectrophotometry that makes it possible to detect enzyme redox status at millisecond time scale. No flavin semiquinone enzyme form was detected, indicating absence of a one-electron transfer event [8]. Electron spin resonance measurements which permit direct detection of Cr(V) conducted with a related enzyme were consistent with these results [9]. Redox balance method indicates that YieF and its evolved enzymes reduce uranyl as well without redox cycling [17].

6. A strategy to mitigate chromate toxicity. Thus bacteria posses "safe" obligatory two electron reducers, and yet chromate generates oxidative stress (Section 4). Clearly the cellular one-electron reducers are more active than the safe enzymes. Would then overproduction in the cell of an enzyme of the safe type mitigate chromate toxicity? The answer is yes: when chromate reduction efficiency per unit growth between the wild type *P. putida*, a mutant missing ChrR, and a strain overproducing this protein

was tested, the mutant showed decreased, and the over-producer increased efficiency of chromate reduction per unit biomass [9].

A strategy therefore presented itself for minimizing chromate toxicity to bacteria and improving their chromate remediation capability: *Improve the kinetics of a safe enzyme for chromate reduction*. We reasoned that a significantly active enzyme containing an appropriately high K_{cat}/K_m would be able to sufficiently circumvent channeling of chromate *in vivo* into the unsafe pathways of one-electron reduction, thereby enabling bacteria to be more resilient in intracellular chromate reduction. This strategy is illustrated in Fig. 3. Indeed, this strategy should improve dissimilatory metal reduction as well, since these bacteria too are subject to oxidative stress by the mechanism described above.

Our studies with uranyl are less extensive so far. But all preliminary indications suggest a situation similar to Cr: aerobic reduction by cells; redox cycling by one-electron reducers; and "safe" reduction by obligatory two-electron reducers [17] (Salles and Matin, unpublished).

7. Directed evolution of YieF. We thus conducted directed evolution of the YieF enzyme to improve its efficiency for chromate reduction followed by high throughput screening [17, 18]. Y6 enzyme is the most effective isolated by this method with a 30-fold greater V_{max} for chromate reduction than YieF as well as other improved characteristics (Table 1). Later studies showed that the evolved enzyme was markedly active also in uranyl reduction (Table 2). We have also used a colorimetric method for a direct high throughput screening of genes encoding improved uranyl reductase activity. Although several have been found, none so far encodes a protein more active in this respect than Y128 and Y150 [18].

Table 1. Kinetics of Cr(VI) reduction of the wild type YieF and the evolved enzymes.

Strain	V_{max} (nmol Cr(VI) mg protein $^{-1}$ min $^{-1}$)	K_m (μM)	K_{cat}* (S^{-1})	K_{cat}/K_m
YieF	295 ± 27	376 ± 14	30 ± 2	$4.5x10^4 \pm 3x10^3$
Y6	8,812 ± 611	41 ± 5	521 ± 18	$1.3x10^7 \pm 3x10^5$
Y150	258,333 ± 16,875	881 ± 353	57,445 ± 3,321	$3x10^7 \pm 1x10^6$

8. Rational approach to further YieF evolution. In collaboration with Dr. Yuval Nov (New York and Haifa Universities), we have also combined directed evolution approach with rational methods of enzyme improvement. Dr. Nov while at Stanford in Dr. Wein's group [19] developed a system for optimizing protein activity under resource constraint, and devised a method to predict improved protein activity based on a limited number of protein variants. Applying his stochastic model relating the sequence and activity of a protein to the 16 mutants of the YieF enzyme involving mutations at 11 positions in the protein and Maximum Likelihood Analysis implemented through MATLAB's optimization toolbox, the 5 two-point mutants with the highest predicted activity were identified and selected to be expressed and screened. All 5 exhibited enhanced activity – on average, higher than the already high average activity of the 16 sequences in the initial data set ($p < 0.01$). One of the five mutants, Y150, showed >830-fold improvement in activity over the wild type with respect to chromate reductase activity, making it the most active chromate reductase known (Tables 1 & 2**;** Barak, Y., Nov, Y., Matin, A. In preparation). This is the enzyme is the main focus of ongoing studies.

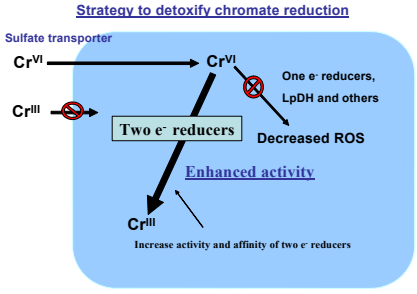

Figure 3. Strategy to minimize chromate toxicity to bacteria – circumventing chromate channeling to one-electron reducers.

9. Evolved enzymes and cancer chemotherapy. Based on the general characteristics of YieF, Y6, and Y150, we tested the latter for prodrug reduction activity and found that they were highly effective in reducing disparate prodrugs, such as mitomycin C, *5-aziridinyl-2,4-dinitrobenzamide* (CB 1954) and the drug *17-Allylamino-17-demethoxygeldanamycin* (17-AAG) [18] (Barak, Y., Matin, A., unpublished). These prodrugs become activated upon reduction and are a highly promising approach to cancer chemotherapy, and thus a side-benefit of this research has been a potentially greatly improved prodrug therapy [18].

10. Amino acid changes underlying improved activity. Y6 exhibited four substitutions, however, reversion studies showed that only one change Tyr^{128} to Asn was responsible for its improved activity. Y150 has an additional substitution, Gly^{150} Ser. That relatively minor changes in the parent enzyme led to marked increases in activity for reducing disparate electrophiles suggests that the amino acid change(s) resulted in amplification of the original enzyme properties, including its broad substrate range, reinforcing the choice of YieF for these studies. They also provide the rationale for the hypothesis we would like to pursue that Y150 is likely to be more active also in reducing other radionuclides. Using the redox balance method and, in collaboration with the Francis group using XANES, we showed that the modification in the enzyme did not alter its characteristic for Cr(III) generation by a simultaneous four-electron chromate reduction [17, 18].

Table 2. Kinetics of U(VI) reduction of the wild type YieF and the evolved Y6 enzymes.

Strain	V_{max} (nmol U(VI) mg protein $^{-1}$ min $^{-1}$)	K_m (μM)	K_{cat}* (S^{-1})	K_{cat}/K_m
YieF	213 ± 17	108 ± 49	29 ± 11	$1.6x10^4 \pm 1.7x10^3$
Y6	2,511 ± 421	779 ± 40	331 ± 39	$5x10^5 \pm 2x10^4$
Y150	4,814 ± 462	221 ± 54	333 ± 37	$7x10^5 \pm 7x10^4$

*Based on dimeric enzyme molecular mass of 50 kDa.

11. Envelope permeability-imposed limitation. We tested the efficacy of Y6 for chromate and uranyl reduction in *P. putida*. The broad host range vector pMMB67EH was used to introduce the YieF- or Y6- encoding genes (*yieF* and *y6*, respectively) into *Pseudomonas* (CRK4 strain). Bacteria over-expressing YieF- or Y6 exhibited only marginally improved chromate reductase activity (Fig. 4A). However, cell extracts of the *yieF*-transformed strain showed higher and those of *y6*-transformed strain even higher chromate reductase activity than the CRK4 extracts (Fig. 4B). The results indicate that the *E. coli* genes *yieF* and its evolved versions were expressed in *P. putida* and suggest that the permeability barrier to chromate masked the enhanced cellular chromate reductase activity of the transformed strains. To further test the involvement of the transport barrier, the capacity of the transformed cells to reduce chromate was determined following their permeabilization by chloroform treatment. The recombinant strains expressing YieF and Y6 now showed greater reductase activity than the non-transformed strain, with the Y6-expressing strain showing the highest activity (Fig 5).

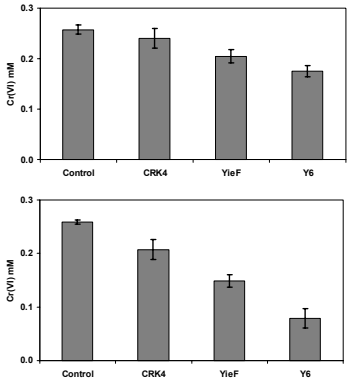

Figure 4. A (upper panle). Cr(VI) conversion by whole cells of *P. putida*, transformed with the empty plasmid, or plasmid containing the *yieF* or *y6* gene. 'Control' refers to chromate conversion in LB medium alone. B (lower panel). Chromate conversion by crude extracts of the above strains.

When the *E. coli* mutant NR698 which is impaired in outer membrane permeability [20] was transformed with these plasmids, the advantage of Y6 in whole cell reduction of chromate again became evident: the mutant expressing this enzyme reduced chromate at a faster rate than the wild type expressing the enzyme. Chloroform-treated MC4100 and NR698 (nonexpressing or overexpressing the cloned YieF or Y6) exhibited a greater reduction rate than untreated NR698 strain, but the difference was only 10-20%. Thus, while cytoplasmic membrane transport barrier also contributed to the masking of Y150 activity, the main barrier was the outer membrane impermeability.

12. Uranyl reduction. Similar results were obtained with uranyl indicating the need to improve envelope permeability to take full advantage of the improved enzyme in enhancing bacterial capacity for uranyl reduction as well. Appropriate controls indicated that uranyl disappearance was not due to trivial reasons such as binding to medium constituents or to the cells [17]. Why was the reduced U not oxidized back to uranyl under the aerobic conditions used is not known. *Whether sequestration of U(IV) within the cell and/or complexation with the medium are possibilities that are being investigated.*.

13. Physiological role of bacterial obligate two-electron reducing oxidoreductases. All of these enzymes also reduced quinones with high efficiency. Just as with chromate, many bacterial enzymes can vicariously reduce quinones by one electron reduction. This generates semiquinone which, like Cr(V), is subject to redox cycling, ROS and oxidative stress generation. Quinones are a constant potential threat to bacteria, being produced by cellular metabolism of aromatics and excreted by plants for defense against

bacterial infections. All three obligate two-electron reducers we examined, YieF, ChrR, and NfsA, were able to outcompete one electron quinone reducers. Absence of ChrR and its overproduction made the cells less and more resistant, respectively, to H_2O_2 than the wild type. Fluorescent-activated cell sorting confirmed that the loss or overproduction of ChrR inside the cell correlates with intracellular levels of H_2O_2. Protein carbonylation, which reflects intracellular oxidative stress, corroborated these results. Thus, the obligate two-electron bacterial oxidoreductases are designed to counter oxidative stress. Evidently, because of their broad substrate range, they can be recruited to minimize oxidative stress from compounds, such as chromate and quinones that have proclivity for one electron reduction [21].

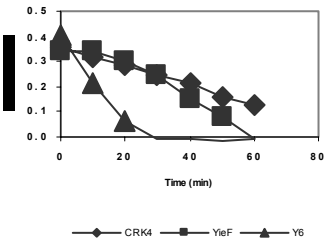

Figure 5. Cr(VI) conversion by whole cells of chloroform-permeabilized *P. putida*, transformed with the empty plasmid (◆), or plasmid containing the *yieF* (■) or *y6* (▲) gene. The experiment was conducted in triplicate runs. Variations between the mean values of each run were less than 10% as determined by the analysis of co-variance.

These findings reinforce two of the premises and objectives of this research, namely that strengthening the activity of these enzymes in the cells will make them more robust and efficient in stressful environments such as the DOE waste sites; and two, that they and their improved variants may be active in also reducing other metals and radionuclides to their stable reduced forms, since many of the latter also are likely to be subject to one-electron reduction.

We have thus attained to a large degree the aim of the research namely evolving highly efficient enzymes that can enhance the capacity of bacteria for remediating multiple metal and radionuclide DOE contaminants while minimizing their toxicity to the bacteria. The stage is now set to explore the possibilities offered by these findings to extend the promise of Y150 type enzymes to: (a) encompass also reduction of additional DOE metals and radionuclides; (b) test the effectiveness of bacteria expressing Y150 to remediate metal and radionuclides in settings that resemble DOE site conditions; and (c) to determine their selective advantage and ecological impact in such settings.

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24. Ackerley,D., and A. Matin. 2004. Chromate-reducing flavoenzymes that guard against oxidative stress. West Coast Bacterial Physiologist Meeting, Asilomar, California

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26. Barak, Y., S. Thorne, D. Ackerley, S. Lynch, C. Contag, and A. Matin. Improved enzymes, generated by DNA shuffling, for reductive cancer chemotherapy. To be presented at AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics. November 14-18, 2005, Philadelphia, PA

27. Francis, A.J., and A.C. Matin. 2006. Molecular mechanisms of uranium reduction by *Clostridia* and its manipulation, DOE NABIR P.I. Workshop, Warrenton, Va. P.50

28. Barak, Y., D. Ackerley, C. Dodge, A. Cheng, Y. Nov, A. Francis, and A. C. Matin. 2006. Generation of a novel high-activity enzyme with combined Cr(VI) and U(VI) reductase activities using directed evolution and rational design. DOE NABIR P.I. Workshop, Warrenton, Va. P.43

29. Yoram Barak, Stephen H. Thorne, Yuval Nov, Christopher H. Contag, Jianghong Rao, and A. Matin. 2006 Improved prodrug chemotherapy through directed enzyme evolution and discovery of a new prodrug. In press, International Journal of Oncology

Other presentations

30. Keynote speaker, "Molecular approaches to improve bacterial bioremediation capacity with special reference to chromate bioremediation", International Symposium of Environemental Biotechnologies on Bioenergy and Bioremediation. National Cheng Kung University, Tainan, Taiwan, September, 2004 (Two additional talks at affiliated Institutes on other aspects of molecular bioremediation)

31. Plenary talk (by A.C. Matin), “Enzyme design for Cr(VI) and U(VI) reduction”, NABIR meeting at Airlie Center, 2006.